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Peptide binding and photo-crosslinking to detergent solubilized and to reconstituted transporter associated with antigen processing (TAP)

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Abstract The transporter associated with antigen processing (TAP) is essential for peptide loading onto major histocompatibility (MHC) class I molecules by translocating peptides into the endoplasmic reticulum. We have explored the conditions for detergent solubilization of functionally active, heterologously expressed human TAP from microsomal membranes. The efficiency to solubilize TAP was tested for a variety of detergents as well as for different solubilization conditions. The activity of the solubilized TAP complex was analyzed over time, using a non-radioactive crosslinking assay with a photo-activateable peptide, in the presence or absence of external lipid. The detergent CHAPS was found optimally to retain activity and thus allowed us to reconstitute detergent-solubilized, active TAP into proteoliposomes.

Key words: ABC transporter; Solubilization; Reconstitution; Membrane protein; Photo-crosslinking

1. Introduction

Cytotoxic T-lymphocytes recognize peptides, derived from endogenous proteins, in association with major histocompatibility complex (MHC) class I molecules. These peptides are generated mainly in the ubiquitin-proteasome degradation pathway and are believed to be translocated into the lumen of the endoplasmic reticulum (ER) by the action of the transporter associated with antigen processing (TAP) for loading onto assembling class I molecules (reviewed in [1–3]). TAP is a heterodimeric protein complex composed of two MHC-encoded subunits, TAP1 and TAP2 [4–7], and belongs to the ATP binding cassette (ABC) superfamily of proteins [8]. These proteins are known for all three kingdoms (domains) of life and are involved in translocation of substrates across membranes. Members include the multidrug resistance P-glycoprotein (MDR), the cystic fibrosis transmembrane conductance regulator (CFTR), and the adrenoleukodystrophy protein (ALDP). They are typically composed of two nucleotide binding domains containing the Walker A/B motifs and the characteristic ABC signature, as well as of two transmembrane domains composed of six to eight membrane spanning regions each. These domains can be found organized on one to four polypeptides. In the case of TAP1 and TAP2 one nucleotide binding domain is linked to one transmembrane domain [9–12]. Four lines of evidence support the idea of the TAP1/TAP2 complex as the peptide translocator in the ER: (i) cytotoxic T-cell recognition could be restored by *tap* transfection of mutant cell lines which are defective in MHC class I-dependent antigen presentation [13–15]; (ii) a functional polymorphism has been reported for rat TAP, affecting the pool of peptides found associated with the same class I molecules [16]; (iii) using streptolysin O-permeabilized cells [17,18] or microsomal membranes [19] ATP-dependent peptide translocation was demonstrated directly by trapping of translocated peptides through *N*-linked glycosylation or MHC class I binding in the ER; and (iv) heterologous expression in insect cells and in yeast demonstrates that the TAP complex is functional in the absence of MHC-encoded factors [7,20]. Functional reconstitution of transport activity for the purified TAP complex would thus be the first direct proof of its function. Here, we report on the exploration of conditions to keep detergent-solubilized TAP in a functional state, as judged by the ability to specifically bind peptides. This is a prerequisite not only for functional reconstitution, but also for attempts to resolve the structure of TAP by spectroscopy or crystallization techniques.

2. Materials and methods

2.1. Cell culture and preparation of microsomes

Spodoptera frugiperda Sf9 cells were grown according to standard procedures and infected with the baculovirus pAcUW51/TAP1-6×His/TAP2, carrying the genes for human 6×His-tagged TAP1 and TAP2 [7]. Microsomes were isolated essentially as previously described [7]. Aliquots of the microsomes were snap frozen in liquid nitrogen and stored at −80°C.

2.2. Peptide synthesis and labeling

Synthesis of the peptides was carried out by solid phase technique on a multiple peptide synthesizer (Syro, MultiSynTech, Bochum, Germany) using Fmoc/tBu (fluorenylmethoxycarbonyl/*tert.*-butyl) chemistry and *p*-benzyloxybenzyl alcohol-PS-DVB (Novabiochem, Läufelingen, Switzerland). The photo-activateable peptide, Fmoc-*p*-(3-trifluoromethyl) diazirinophenylalanine, was kindly provided by Dr. J. Brunner (ETH Zürich, Switzerland), and coupled manually as described, using a threefold molar excess and a coupling time of 20 h [21]. The 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) protection group for lysine was cleaved with 2% hydrazine in dimethylformamide (5×3 min). Manual coupling of Fmoc-6-aminohexanoic acid (ahx) was performed, using a 10-fold excess and a coupling time of 16 h. After deprotection, biotinylation was achieved with a twofold molar excess of EZ-Link *N*-hydroxysuccinimide (NHS)-biotin (Pierce) in

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Abbreviations: BIGCHAP, *N,N*-bis[3-D-gluconamidopropyl]-cholamide; $C_{12}E_9$, nonaethylene glycol monododecyl ether; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, 1,4-dithio-DL-threitol; MEGA 9, nonanoyl-*N*-methylglucamide; MEGA 10, decanoyl-*N*-methylglucamide; NP40, nonaethylene glycol octylphenyl ether; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; sulfobetaine, *N,N*-dimethyl-3-ammino-1-propane-sulfonate; Tween 80, polyoxyethylene sorbitan monooleate

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dimethylformamide, overnight. The purity and identity of the peptide was verified by reversed phase HPLC and mass spectrometry. Radiolabeling of RRYQKSTEL was performed as described before [7] according to the method of Hunter and Greenwood [22]. The reaction was performed with 8 nmol of peptide and 1.85×10^7 Bq (0.5 mCi) $Na^{125}I$. Free iodine was removed by gel filtration on a Sephadex G10 column (Pharmacia). The specific activity was 53 cpm/fmol.

2.3. Solubilization and immunodetection

Microsomes containing 2% functional TAP complexes from the total protein [23] and an equal lipid-to-protein ratio (w/w) were washed once in solubilization buffer (140 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 15% glycerol, 1 mM DTT, 20 mM HEPES-NaOH, pH 7.4) and resuspended at 8 mg/ml of protein corresponding to 10 mM lipid in same buffer supplemented with the appropriate detergent concentration, for 20 min on ice, unless indicated otherwise. Insoluble material was removed by centrifugation for 20 min at $104\,000\times g$ at 4°C. For SDS-PAGE and immunodetection, the supernatant was precipitated according to the chloroform/methanol method of Wessel and Flügge [24] to remove excess detergent and lipid. Samples were run on a 8–18% gradient SDS/polyacrylamide gel [25] and electroblotted onto nitrocellulose. Immunodetection was as indicated, using either a mixture of the anti-TAP1 (148.3) [7] and anti-TAP2 (435.3) [26] monoclonals or a polyclonal antibody against biotin (Rockland Inc., Pennsylvania, USA) and horseradish peroxidase-coupled secondary antibodies in combination with an enhanced chemiluminescence Western blotting detection system (Amersham) as recommended by the manufacturer.

2.4. Photo-crosslinking and detection

Photo-crosslinker peptide was added to solubilized microsomal proteins, or to reconstituted proteoliposomes (see Section 2.5), to yield a saturating final concentration of 800 nM. UV irradiation was performed in ice-cooled aluminum devices for 3 min at 350 nm with a 210 W UV lamp (Fluotest forte, Xenotest, Germany). Proteoliposomes were washed in phosphate buffered saline and solubilized in solubilization buffer/25 mM CHAPS at ρ=2 (see Section 3.1). In order to reduce non-specific binding of the anti-biotin polyclonal antibody during immunodetection, the TAP1/TAP2 complex was enriched by metal chelate affinity chromatography performed in batch, but otherwise as described previously [7]. Eluted protein was subjected to chloroform/methanol precipitation before SDS-PAGE and immunodetection as in Section 2.3.

2.5. Reconstitution and peptide binding

To a mixture of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol (6:2:1:1, Sigma), a two-fold molar excess of CHAPS over lipid was added, and the mixture was dried under a stream of nitrogen and in vacuum. After the addition of phosphate buffered saline to yield a lipid concentration of 10 mg/ml, the lipid-detergent film was dissolved by gentle shaking. Insoluble material was removed by centrifugation at $104\,000\times g$ for 20 min. 5 mg of microsomal protein was solubilized in solubilization buffer/25 mM CHAPS at ρ = 2 (see Section 3.1). After removal of insoluble material by centrifugation, 10 mg of CHAPS-solubilized lipid was added and the mixture was dialyzed against solubilization buffer for 3 h and subsequently against phosphate buffered saline/1 mM DTT for additional 5 h. Proteoliposomes were collected by centrifugation at $104\,000\times g$, 4°C for 20 min and resuspended in phosphate buffered saline/1 mM DTT. Then, proteoliposomes were used for either photo-crosslinking (see Section 2.4) or peptide binding assays with the radiolabeled peptide *RRYQKSTEL*. Aliquots were incubated with 100 nM peptide for 5 min on ice, in the presence or absence of a 200-fold molar excess of competitor, as indicated. Unbound peptide was removed by centrifugation and bound peptide was quantified, after one washing step, by γ-counting.

Table 1
Solubilization efficiency of various detergents

Detergent	ρ = 2	ρ = 5
$C_{12}E_9$	> 95%	> 95%
NP40	> 95%	> 95%
Tween 80	28%	33%
n-Octyl glucoside	< 5%	< 5%
n-Octyl thioglucoside	< 5%	< 5%
MEGA 9	40%	73%
MEGA 10	< 5%	< 5%
n-Decyl maltoside	> 95%	> 95%
n-Dodecyl maltoside	> 95%	> 95%
n-Dodecyl sulfobetaine	> 95%	> 95%
CHAPS	35%	38%
BIGCHAP	9%	12%
Digitonin	25%	33%

TAP (2% of the total protein, w/w) was solubilized from insect cell microsomes, using different detergents at two relative effective concentrations (see text) and a protein concentration of 8 mg/ml, corresponding to a lipid concentration of 10 mM. The solubilization efficiency was estimated from densitometric scans of immunoblots of solubilized vs. non-solubilized TAP.
Values are averages of multiple experiments.

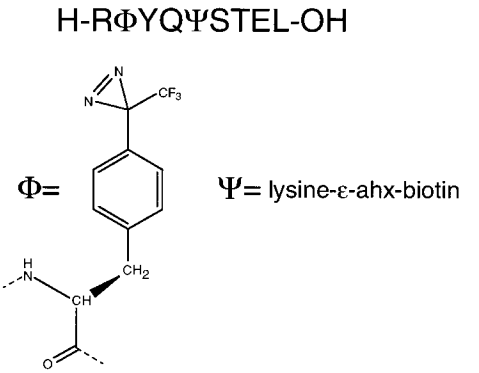

Fig. 1. Structure of the photo-activateable peptide used for non-radioactive crosslinking. The biotin moiety is attached to lysine via a 6-aminohexanoic acid (ahx) spacer.

3. Results and discussion

3.1. Detergent solubilization of TAP

Efficient solubilization is an important step for the purification of a membrane protein. In order to identify solubilization conditions for the TAP complex, we screened various neutral or zwitterionic detergents, compatible with most chromatographic procedures, at two distinct concentrations (Table 1). For an easier comparison of detergents with differences in their critical micellar concentration (cmc), the relative amounts of detergent used are given as the effective relative detergent amount above the cmc, ρ, with ρ defined as $(\text{conc.}_{\text{detergent}}-\text{cmc})/\text{conc.}_{\text{lipid}}$ [27]. The detergents tested could be classified into three classes: (i) non-solubilizing detergents, including *n*-octyl glucoside, *n*-octyl thioglucoside and MEGA 10, (ii) weakly solubilizing detergents, including cholate-based detergents, such as CHAPS, BIGCHAP and digitonin, as well as Tween 80 and MEGA 9, and (iii) detergents that solubilize nearly quantitatively, including decyl and dodecyl maltoside, dodecyl sulfobetaine and the polyoxyethylene ether detergents, NP40 and $C_{12}E_9$. For CHAPS, we tested different solubilization conditions, varying concentration, temperature, pH, salt concentration and time, albeit with no significant improvement (data not shown). Thus, optimal solubilization with CHAPS and the other detergents could already be achieved at a relatively low excess of detergent, after 20 min, thereby making it possible to minimize putative inactivation. Unfortunately, solubilization with the relatively mild detergent CHAPS could not be optimized to levels comparable to those for decyl maltoside or NP40. This is possibly due

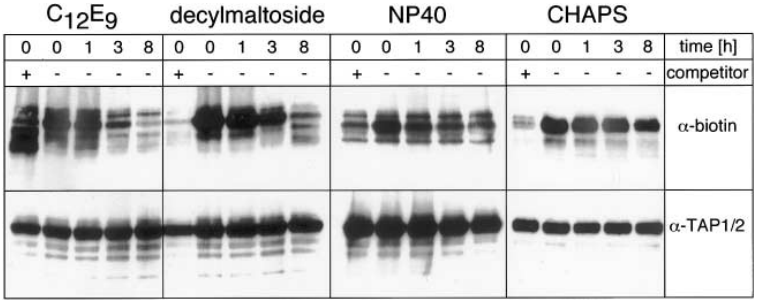

Fig. 2. Inactivation kinetics of TAP, solubilized in different detergents. TAP-containing microsomes from insect cells were solubilized in $C_{12}E_9$, CHAPS, decyl maltoside and NP40 at 20 mM detergent and $\rho = 2$. Photo-crosslinking was performed in the absence or presence of a 200-fold molar excess of competitor peptide at various time points in order to study the inactivation of TAP by the detergents. Detection was after SDS-PAGE and immunoblotting with anti-biotin or anti-TAP1/2 antibodies.

to the lipid composition of the insect cell ER, since CHAPS is known to be particularly efficient in disrupting membranes with a high cholesterol content [28].

3.2. Stability of TAP in various detergents

Measuring transport activity of membrane proteins at different stages from a protein purification protocol, and thus in the detergent-solubilized state, is intrinsically difficult. We circumvented this problem by taking advantage of the fact that TAP binds peptides reversibly, specifically, and with a high affinity at low temperatures [23,26,29,30], allowing photo-crosslinking to be performed with TAP [31,32]. We could thus compare the different detergents that have proven to be efficient at solubilization, with respect to their effect on TAP peptide binding activity over time, concentrating on four selected detergents. Decyl maltoside and CHAPS were preferred over dodecyl maltoside and digitonin due to their higher cmc. The readout was through the amount of photo-activateable peptide that could be specifically crosslinked to TAP. Since the photo-crosslinker peptide used was bifunctional, carrying a biotin for detection, we were able to set up a non-radioactive detection system by immunoblotting, using a polyclonal anti-biotin antibody (Fig. 1). We found that the peptide binding function was partially retained in the solubilized state for the detergents tested. The inactivation of peptide binding to TAP was fastest with $C_{12}E_9$ and NP40: most of the activity was lost after 3 h and was nearly completely abolished after 8 h (Fig. 2). Using decyl maltoside, again most of the activity was lost after 8 h, but it could be largely retained over the first 3 h. With CHAPS even after 8 h a considerable residual activity could be detected. In order to correct for the different solubilization efficiencies of the detergents used and to rule out day-to-day variations, photo-crosslinking was performed in parallel, using equal amounts of solubilized TAP for the four detergents (Fig. 3). Initial inactivation was found to be strongest for $C_{12}E_9$ and NP40 again, with CHAPS and decyl maltoside retaining activity equally well, relative to protein levels. Addition of external lipid is sometimes used to prevent inactivation due to detergent-dependent delipidation of membrane proteins [33]. Thus, we also tested the effect of added lipid on peptide binding after 8 h. Using $C_{12}E_9$ and NP40, activity was lost nearly completely and could only be restored to a small extent by addition of lipid for $C_{12}E_9$. Also, for decyl maltoside and CHAPS, no beneficial effect could be observed in the presence of additional lipid, although residual activity was relatively high, especially for CHAPS. We conclude that the peptide binding activity of TAP is best preserved in the detergent-solubilized state using CHAPS, followed by decyl maltoside, while the other detergents tested appear to be not suitable for purification and reconstitution experiments. CHAPS is particularly well suited for the retention of activity and is our preferred detergent, despite its lower efficiency for solubilization. Another option would be the use of a detergent mixture of CHAPS and decyl maltoside for solubilization, followed by the use of only CHAPS in subsequent purification and reconstitution procedures. This would combine the efficient solubilization by decyl maltoside with the superior effect of CHAPS on TAP activity.

3.3. Incorporation of solubilized TAP into proteoliposomes

In order to test the suitability of CHAPS-solubilized TAP for reconstitution into proteoliposomes, as well as to test the integrity of TAP after solubilization in more detail, we incorporated TAP into proteoliposomes by dialysis. Using these proteoliposomes, we performed peptide binding experiments with radiolabeled *RRYQKSTEL*, in analogy to our studies

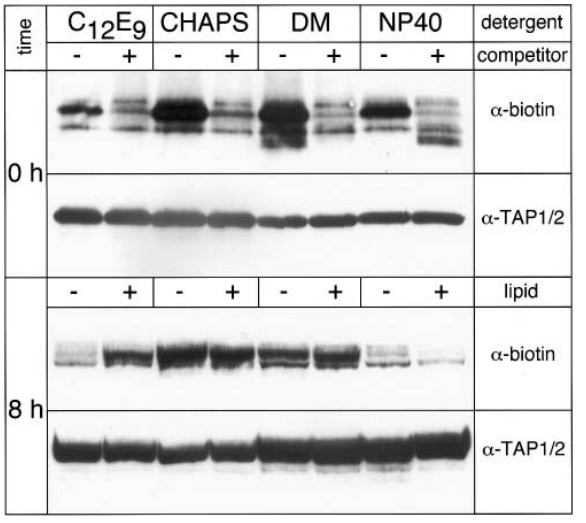

Fig. 3. Initial activity, inactivation and rescue of TAP activity by addition of lipid. In order to compare the initial activity of the solubilizied TAP complex in various detergents, photo-crosslinking was performed. After 8 h, residual activity of the TAP complex as well as the beneficial effect of added external lipid (5 mg/ml) were assayed. The photo-crosslinked TAP was assayed by SDS-PAGE and immunoblotting using an anti-biotin antibody. Equal amounts of TAP were used in each assay, as demonstrated by re-probing the immunoblot by anti-TAP1/2 antibodies.

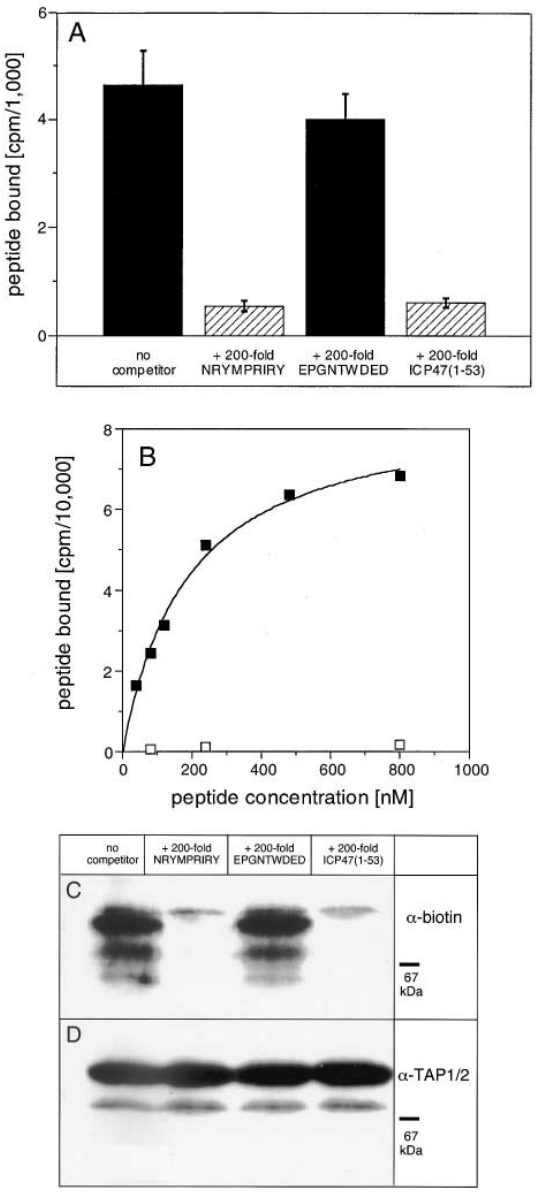

Fig. 4. A: Photo-crosslinking and binding of radiolabeled peptide to TAP-containing proteoliposomes. Proteoliposomes were formed from solubilized microsomal protein after the addition of lipid and dialysis for 8 h. Binding of the radiolabeled peptide *RRYQKSTEL* to the proteoliposomes in the absence and presence of competitors was as described in Section 2. B: The competitors used are: the high affinity peptide NRYMPRIRY, the low affinity peptide EPGNTWED and the TAP-specific inhibitor ICP47(1–53). Additionally, a saturation binding experiment was performed with the radiolabeled peptide *RRYQKSTEL* in the absence (closed symbols) and presence (open symbols) of a 200-fold molar excess of unlabeled RRYQKSTEL. C: Photo-crosslinking was performed with proteoliposomes, using the same set of competitors. D: The immunoblot was reprobed, using an anti-TAP antibody mixture, to confirm that equal amounts of TAP were used. Note that (A) and (B) are from separate experiments with different amounts of TAP reconstituted into proteoliposomes.

using TAP-containing microsomes [23] (Fig. 4A,B). Peptide binding could be inhibited by competition with the peptide NRYMPRIRY, a peptide designed for high affinity binding to TAP [30], but not with the low affinity peptide EPGNTWDED. Additionally, a specific inhibitor for human TAP, ICP47(1–53), derived from herpes simplex virus protein ICP47 interfering with antigen presentation [34,35], was effective at competition, indicating that not only the peptide binding pocket of TAP is preserved but also the overall structure required for interaction with the inhibitor. A saturation binding experiment with radiolabeled peptide revealed that binding affinity was fully retained ($K_D = 167 \pm 47$ nM, as compared to 146 ± 23 nM from Scatchard analysis using microsomes [23]). When photo-crosslinking was performed using these proteoliposomes, a similar pattern was observed for the competitors as in the radio-ligand binding assay (Fig. 4C,D). Taken together, our results indicate that the detergent CHAPS is suitable for solubilization, retention of activity over the time required for purification and can be used for incorporation of functional TAP into proteoliposomes. This will enable us to use purified and functionally reconstituted TAP for mechanistic studies on peptide translocation, which are hampered by the complexity of the microsomal system, as well as for attempts of crystallization of TAP.

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References

[1] Androlewicz, M.J. and Cresswell, P. (1996) Immunity 5, 1–5.
[2] Koopmann, J.O., Hämmerling, G.J. and Momburg, F. (1997) Curr. Opin. Immunol. 9, 80–88.
[3] Tampé, R., Urlinger, S., Pawlitschko, K. and Uebel, S. (1997) in: Unusual Secretory Pathways: From Bacteria to Man (Kuchler, K., Rubartelli, A. and Holland, B., Eds.), pp. 115–136, Springer, New York.
[4] Spies, T., Cerundolo, V., Colonna, M., Cresswell, P., Townsend, A. and DeMars, R. (1992) Nature 355, 644–646.
[5] Kelly, A.P. et al. (1992) Nature 355, 641–644.
[6] Kleijmeer, M., Kelly, A., Geuze, H.J., Slot, J.W., Townsend, A. and Trowsdale, J. (1992) Nature 357, 342–344.
[7] Meyer, T.H., van Endert, P.M., Uebel, S., Ehring, B. and Tampé, R. (1994) FEBS Lett. 351, 443–447.
[8] Higgins, C.F. (1992) Annu. Rev. Cell Biol. 8, 67–113.
[9] Monaco, J.J., Cho, S. and Attaya, M. (1990) Science 250, 1723–1726.
[10] Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A. and Kelly, A. (1990) Nature 348, 741–744.
[11] Spies, T., Bresnahan, M., Bahram, S., Arnold, D., Blanck, G., Mellins, E., Pious, D. and DeMars, R. (1990) Nature 348, 744–747.
[12] Deverson, E.V., Gow, I.R., Coadwell, W.J., Monaco, J.J., Butcher, G.W. and Howard, J.C. (1990) Nature 348, 738–741.
[13] Spies, T. and DeMars, R. (1991) Nature 351, 323–324.
[14] Powis, S.J., Townsend, A.R.M., Deverson, E.V., Bastin, J., Butcher, G.W. and Howard, J.C. (1991) Nature 354, 528–531.
[15] Attaya, M. et al. (1992) Nature 355, 647–649.
[16] Powis, S.H., Mockridge, I., Kelly, A., Kerr, L.A., Glynne, R., Gileadi, U., Beck, S. and Trowsdale, J. (1992) Proc. Natl. Acad. Sci. USA 89, 1463–1467.
[17] Neefjes, J.J., Momburg, F. and Hämmerling, G.J. (1993) Science 261, 769–771.
[18] Androlewicz, M.J., Anderson, K.S. and Cresswell, P. (1993) Proc. Natl. Acad. Sci. USA 90, 9130–9134.
[19] Shepherd, J.C., Schumacher, T.N., Ashton-Rickardt, P.G., Imaeda, S., Ploegh, H.L., Janeway, C.A.J. and Tonegawa, S. (1993) Cell 74, 577–584.
[20] Urlinger, S., Kuchler, K., Meyer, T.H., Uebel, S. and Tampé, R. (1997) Eur. J. Biochem. 245, 266–272.
[21] Weber, P.J.A. and Beck-Sickinger, A.G. (1997) J. Peptide Res. 49, 375–383.

[22] Hunter, W.M. and Greenwood, F.C. (1962) Nature 194, 495–496.
[23] Uebel, S., Meyer, T.H., Kraas, W., Kienle, S., Jung, G., Wiesmüller, K.H. and Tampé, R. (1995) J. Biol. Chem. 270, 18512–18516.
[24] Wessel, D. and Flügge, U.I. (1984) Anal. Biochem. 138, 141–143.
[25] Laemmli, U.K. (1970) Nature 227, 680–685.
[26] van Endert, P.M., Tampé, R., Meyer, T.H., Tisch, R., Bach, J.F. and McDevitt, H.O. (1994) Immunity 1, 491–500.
[27] Rivnay, B. and Metzger, H. (1982) J. Biol. Chem. 257, 12800–12808.
[28] Schürholz, T. (1996) Biophys. Chem. 58, 87–96.
[29] van Endert, P.M., Riganelli, D., Greco, G., Fleischhauer, K., Sidney, J., Sette, A. and Bach, J.F. (1995) J. Exp. Med. 182, 1883–1895.
[30] Uebel, S., Kraas, W., Kienle, S., Wiesmüller, K.-H., Jung, G. and Tampé, R. (1997) Proc. Natl. Acad. Sci. USA 94, 8976–8981.
[31] Androlewicz, M.J. and Cresswell, P. (1994) Immunity 1, 7–14.
[32] Nijenhuis, M., Schmitt, S., Armandola, E.A., Obst, R., Brunner, J. and Hämmerling, G.J. (1996) J. Immunol. 156, 2186–2195.
[33] Callaghan, R., Berridge, G., Ferry, D.R. and Higgins, C.F. (1997) Biochim. Biophys. Acta 1328, 109–124.
[34] Beinert, D., Neumann, L., Uebel, S. and Tampé, R. (1997) Biochemistry 36, 4694–4700.
[35] Neumann, L., Kraas, W., Uebel, S., Jung, G. and Tampé, R. (1997) J. Mol. Biol. 272, 484–492.